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(71) Applicant: UNIVERSITY OF MIAMI [US/US]; 1400 N.W. 10th Avenue, Miami, FL 33101 (US).

(72) Inventors: PFLUGFELDER, Stephen, C.; 9945 S.W. 57th Court, Miami, FL 33156 (US). YOSHINO, Kenichi; 7730 Camino Real, Miami, FL 33143 (US). TSENG, Scheffer, C., G.; 10000 S.W. 63rd Place, Miami, FL 33156 (US). HUANG, Andrew, J., W.; 5845 S.W. 100th Street, Miami, FL 33156 (US).

(74) Agents: LESTER, Michelle, N. et al.; Cushman, Darby & Cushman L.L.P., 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(54) Title: PRODUCTS OF LACRIMAL GLAND FOR TEAR REPLACEMENT

(57) Abstract

The present invention relates to medicinal compositions and more particularly refers to such compositions for tear replacement therapy having products of human lacrimal gland acinar epithelia, and more specifically, growth factors or cytokines, in particular, the transforming growth factor beta (TGFβ).

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TITLE

Products of Lacrimal Gland for Tear Replacement.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to medicinal compositions and more particularly refers to such compositions for tear replacement therapy having products of human lacrimal gland acinar epithelia, and more specifically, growth factors or cytokines, in particular, the transforming growth factor beta (TGFß).

2. Background information

Aqueous tear deficiency is a common condition that in its most severe form may be associated with disabling ocular irritation, and visual morbidity due to corneal epitheliopathy and/or ulceration. The conjunctival pathology of Sjogren's Syndrome (SS), the most severe type of aqueous tear deficiency, consists of abnormal terminal differentiation with significantly reduced bulbar goblet cell densities (Pflugfelder, S.C. et al. Ophthalmology 1990;97:985-991), decreased expression of mucins by the superficial epithelium (Table I)(Pflugfelder, S.C. et al. 1994 ARVO abstracts. Invest. Ophthalmol. Vis. Sci. 1994; 34: 1692)), and aberrant expression of immune activation markers (HLA Class II antigens and ICAM I) and interleukin 6 (IL-6) (Jones, D.T. et al. Invest. Ophthalmol. Vis. Sci. (in press)).

TABLE I

Results of Immunohistochemical Staining of Bulbar Conjunctival Epithelial Cells on Impression Cytology Specimens using Mucin-Specific Antibody L6

Group (%+)	Temporal Conjuctiva (%+)	Inferior Conjuctiva
Sjogren Syndrome (SS) ATD	18.2	18.2
Non Sjogren Syndrome ATD	66.7	88.9
inflammatory MGD	77.8	88.9
Atrophic MGD	77.8	100
Control	100	100

SS vs Inflam. MGD p=0.022 SS vs non SS ATD p=0.005
SS vs Atrophic MGD p=0.022 SS vs Inflam. MGD p=0.005
SS vs control p=0.001 SS vs Atrophic MGD p=0.001
SS vs control p=0.001

ATD = aqueous tear deficiency, MGD = meibomian gland disease

At the present time, biological activity of tears on the health and differentiation of the ocular surface epithelia has not been evaluated. Clinical signs and ocular surface pathologic changes in patients with aqueous tear deficiency suggest that the tears may have more than a lubricating role for the ocular surface. One of the most specific clinical signs of severe aqueous tear deficiency is staining of the conjunctival and/or cornea with the diagnostic dye rose-bengal. Recently reported experimental evidence suggests that rose-bengal staining of the ocular surface epithelia may result from lack of cell coating by normal tear constituents, predominantly tear mucins (Feenstra, R.P. and Tseng, S.C.G. Arch. Ophthalmol. 1992;110:984-993). Mucin-producing goblet cells and production of cell-membrane associated mucins by the superficial stratified epithelia are markers of terminal differentiation in the normal human conjunctiva. A marked reduction in expression of

both types of conjunctival mucin has been detected in the conjunctival epithelia of Sjogren's Syndrome patients (Pflugfelder, S.C. et al. Ophthalmology 1990;97:985-991. Pflugfelder, S.C. et al., 1994 ARVO abstracts Invest. Ophthalmol. Vis. Sci. 1994; 34: 1692).

Although this may be due in part to mechanical trauma related to the reduced preocular tear film, it may also represent abnormal terminal differentiation due to lack of biologically active tear constituents. At the present time, epidermal growth factor (EGF) is the only cytokine that has been detected in human tears (van Seten, G.B. et al. Graeffe's Arch. Clin. Exp. Ophthalmol. 1989;227: 184-187). Reduced tear EGF concentrations have been reported in one patient with aqueous tear deficiency (van Seten, G.B. et al. Curr. Eye Res. 1991; 10:523-527; however, the biologic activity of tear EGF has not been evaluated.

Tear secretion by the human lacrimal gland is influenced by neurotransmitters and hormones (Dartt, D. Curr. Eye Res. 1989;8:619-636; Sullivan, D.A. The Neuro Endocrine-immune Network S. Freier, Editor. Boca Raton, FL 1990 CRC Press, pp 199-238). Jordan and Baum have reported that the majority of tear secretion is reflexive, resulting from sensory stimulation of the lids and ocular surface (Jordan, A. and Baum, J. Ophthalmology 1980;87:920-930). A marked reduction in neural-stimulated tear secretion is an early clinical sign in Sjogren's Syndrome (Tsubota, K. Am. J. Ophthalmol. 1991; 111: 106-108), but the clinical consequences of reduced neural-stimulated tears have not been established.

We recently discovered that the pathologic changes associated with Sjogren's Syndrome may be due in part to reduced concentrations of cytokines produced by the lacrimal gland and secreted into the tears that are essential for normal health and differentiation of the ocular surface epithelia. Based on its ability to induce differentiation of intestinal mucosa (Kurokowa, M. et al., Biochem. Biophys. Comm. 1987; 142:775-782), and corneal epithelia (Kruse, F.E. and Tseng. S.C.G. Invest. Ophthalmol. Vis. Sci. 1993;34: 1963- 1976), and its ability to down regulate HLA Class II antigen and IL-6 expression (Lucas, C. et al. Ciba Foundation 1991; 157:98- 114), we hypothesized that transforming growth factor beta (TGFß) may be one of the biologically essential tear cytokines. Recently, TGF has been reported to be produced by mammary gland acini (Maier, R. et al. Mol. Cell. Endocrinol. 1991 ;82: 192-198) and secreted into milk.

TGFß is a multi-functional biologically essential cytokine. TGFß has a spectrum of biologic activity and has been reported to induce differentiation and inhibit proliferation of mucosal epithelia, including rabbit corneal epithelia (Kurokowa, M. et al. Biochem. Biophys. Comm. 1987; 142:775-782; Kruse, F.E. and Tseng, S.C.G. Invest. Ophthalmol. Vis. Sci. 1993;34: 1963-1976).
TGFß has also been reported to stimulate synthesis of extra cellular matrix components and has been shown to induce these effects on corneal stromal fibroblasts (Ohji, M. et al. Curr. Eye. Res. 1993;12:703-709). Finally, TGFß has immunosuppressive activity that includes inhibition

of T-cell proliferation, down regulation of expression of inflammatory cytokines such as IL-6 and immune activation markers such as HLA class II antigens (Lucas, C. et al. Ciba Foundation 1991; 157:98- 114).

At the present time, commercially available artificial tear replacements are composed of synthetic polymers, buffers, and electrolytes in an aqueous solution. Examples of such solutions include "BION" (Alcon Laboratories, Fort Worth, TX) and "REFRESH PLUS" (Allersan, Irvine, CA). Major components of commercially available artificial tear replacement solutions, Ophthalmic lubricants which protect the eye from drying, and ocular decongestants, are listed in TABLES II, III, and IV, respectively. These solutions contain no biologically active components to modulate the health and differentiation of ocular surface epithelia. Tear replacement therapies containing biologically active components could potentially reverse pathologic ocular surface epithelial changes, and would present a great advance in treatment of severe aqueous tear deficiency states.

TABLE II

ARTIFICIAL TEAR PREPARATIONS

MAJOR COMPONENT	CONCENTRATION	TRADENAME	PRESERVATIVE/EDTA
Carboxy methylcellulose	0.5%	Cellufresh	None
	1%	Celluvisc	None
Hydroxyethyl cellulose		Lyteers	Benzalkonium Cl + EDTA
		TearGard	Sorbic Acid + EDTA
Hydroxyethyl cellulose + Polyvinyl Alcohol		Neo-Tears	Thimerosal + EDTA
Pydroxyethyl cellulose + Povidone		Adsorbotear	Thimerosal + EDTA
Hydroxypropyl Cellulose		Lacrisert (Biode-gradable insert)	None
Hydroxypropyl Methylcellulose	0.5%	Isopto Plain	Benzalkonium Cl
		Isopto Tears	Benzalkonium Cl
		Tearisol	Benzalkonium Cl + EDTA
	1%	Isopto Alkaline	Benzalkonium Cl
		Ultra Tears	Benzalkonium Cl
Hydroxypropyl Methylcellulose + Dextran 70		Tears Naturale	Benzalkonium Cl + EDTA
		Tears Naturale II	Polyquad
		Tears Naturale Free	None
Hydroxypropyl Methylcellulose + Gelatin A		Lacril	Chlorobutanol + Polysorbate 80
Methylcellulos	1%	Murocel	Methyl- + Propylparabens
Polyvinyl Alcohol	1.4%	Akwa Tears	Benzalkonium Cl + EDTA
		Just Tears	Benzalkonium Cl + EDTA
		Liquifilm Tears	Chlorobutanol
	3%	Liquifilm Forte	Thimerosal + EDTA
Polyvinyl Alcohol + PEG-400 + Dextose	1%	Hypotears	Benzalkonium Cl + EDTA
		Hypotears PF	EDTA
Polyvinyl Alcohol + Povidone	1.4%	Murine	Benzalkonium Cl + EDTA
	0.6%	Refresh	None
		Tears Plus	Chlorobutanol

TABLE III

OPHTHALMIC LUBRICANTS

TRADE NAME	COMPOSITION
AKWA Tears Ointment (Akorn)	Sterile ointment containing white petrolatum, liquid lanolin, and mineral oil.
Duolube (Bausch & Lomb)	Sterile ointment containing white petrolatum and mineral oil.
Duratears Naturale (Alcon)	Sterile ointment containing white petrolatum, liquid lanolin, and mineral oil.
HypoTears (Iolab)	Sterile ointment containing white petrolatum and light mineral oil.
Lacri-Lube S.O.P. (Allergan)	Sterile ointment containing 42.5% mineral oil, 55% white petrolatum, lanolin alcohol, and chlorobutanol.
Refresh P.M. (Allergan)	Sterile ointment containing 41.5% mineral oil, 55% white petrolatum, petrolatum, and lanolin alcohol.

TABLE IV

OCULAR DECONGESTANTS

DRUG	TRADE NAME	ADDITIONAL COMPONENTS
Naphazoline Hydrochloride	AK-Con*	Benzalkonium Cl + edetate disodium
	Albalon*	Benzalkonium Cl + edetate disodium
	Clear Eyes	Benzalkonium Cl + edetate disodium
	Degest 2	Benzalkonium Cl + edetate disodium
	Naphcon*	Benzalkonium Cl + edetate disodium
	Opcon*	Benzalkonium Cl + edetate disodium
	Vasoclear	Benzalkonium Cl + edetate disodium
	Vasocon Regular*	Phenylmercuric acetate
Phenylephrine Hydrochloride	AK-Nefrin	Benzalkonium Cl + edetate disodium
	Efricel	Benzalkonium Cl + edetate disodium
	Eye Cool	Thimerosal + edetate disodium
	Isopto Frin	Benzalkonium Cl + edetate disodium
	Prefrin Liquifilm	Benzalkonium Cl + edetate disodium
	Relief	--
	Tear-Efrin	Benzalkonium Cl + edetate disodium
	Velva-Kleen	Thimerosal + edetate disodium
Tetrahydrozoline Hydrochloride	Collyrium	Benzalkonium Cl + edetate disodium
	Murine Plus	Benzalkonium Cl + edetate disodium
	Soothe*	Benzalkonium Cl + edetate disodium
	Tetracon	Benzalkonium Cl + edetate disodium
	Visine	Benzalkonium Cl + edetate disodium
DECONGESTANT/ASTRINGENT COMBINATIONS		
Naphazoline Hydrochloride plus Zinc Sulfate	Clear Eyes ACR (Allergy/Cold Relief)	Benzalkonium Cl + edetate disodium
Phenylephrine Hydrochloride plus Zinc Sulfate	Prefrin-Z	Thimerosal
	Zincfrin	Benzalkonium Cl
Tetrahydrozoline plus Zinc Sulfate	Visine A.C.	Benzalkonium Cl + edetate disodium

* Prescription medication

SUMMARY OF THE INVENTION

We have recently been able to culture human lacrimal gland acinar epithelia which secrete proteins typically produced by lacrimal gland secretory acini in vivo (Yoshino, K. et al., Proceedings of the Fourth International Symposium on Sjorgren's Syndrome (1993), p. 804). In addition, we have evaluated human tears for TGFß using the CCL-64 mink lung epithelial cell (MLEC) growth inhibition assay and sELISA. Results indicate that human lacrimal gland acini produce and secrete TGFß into the tears, and that there are factors in human tears capable of binding TGFß.

It is therefore an object of the present invention to provide cultured human lacrimal gland acinar epithelia as a model of *in vivo* secretory acinar function. These cultures can be used for testing of agents which stimulate or inhibit tear secretion and the analysis of biologically active tear constituents that are secreted by the lacrimal gland which can be used for the treatment of diseases affecting the ocular epithelia. Specifically, diseases of the ocular surface associated with aqueous tear deficiency.

It is another object of the present invention to provide a medicinal formulation suitable for the treatment of various conditions which result in tear deficiency or ocular irritation. Conditions benefiting from physiologic tear replacement include patients with lacrimal gland dysfunction, destruction or surgical removal (Sjogren's Syndrome, post radiation, altered innervation, surgical removal for treatment of tumor).

It is yet another object of the invention to provide tear replacement compositions containing TGFß which are more effective than the composition presently in use which do not contain biologically active components. According to the present invention, tear replacement compositions are provided by adding TGFß to a pharmaceutical composition for application to the eye in order to lubricate the eye or to supplement tears.

According to the present invention, tear replacement compositions as stated above may also contain any or other components produced by lacrimal gland epithelia, naturally present in human tears such as antimicrobial proteins (for example lactoferrin and lysozyme), retinol binding protein (for example tear specific pre-albumin), biologically active components or cytokines such as epidermal growth factor, or retinol.

Compositions according to the present invention can be used to treat aqueous tear deficiency and conditions associated with alterations of the ocular suface epithelia including hyperproliferation, squamous metaplasia, loss of goblet cells, and abnormal terminal differentiation among other ocular surface pathologic changes that lead to ocular irritation.

The foregoing and other objects, advantages and characterizing features of this invention will become apparent from the following description of certain illustrative embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A. Expression of TGFß1 mRNA in normal human lacrimal gland biopsies and cultured human lacrimal gland acinar epithelia. PCR products of the appropriate size (161 bp) from amplification of cDNA prepared from human lacrimal gland epithelial cultures (lane 1) and human lacrimal gland biopsies (lane 2-4) with TGF-ß1 specific primers were noted on an ethidium bromide stained agarose gel (upper figure) and Southern hybridization (bottom figure). Lane 5 contains TGF-ß1 cDNA. Lane 6- blank, Lane 7- molecular weight standards.

Figure 1B. Expression of TGFß2 mRNA in normal human lacrimal gland biopsies and cultured human lacrimal gland acinar epithelia. A PCR product of approximately 450 bp was noted on an ethidium bromide stained agarose gel of amplification of cDNA prepared from human lacrimal gland epithelial cultures (lane 1) and human lacrimal gland biopsies (lanes 2-4) with TGF-ß2 specific primers. On Southern hybridization, three hybridizations signals with approximate sized of 350-, 450-, and 500-bp were obtained from cDNA prepared from cultured lacrimal gland epithelia (lane 1) and one lacrimal gland biopsy (lane 2), while only two hybridization bands (350 and 450 bps) were obtained from cDNA prepared from the other two lacrimal gland biopsies (lanes 3 and 4). Multiple sized PCR products are most likely due to alternate splicing of the region of the TGF-ß2 gene amplified with these primers. Lane 5 contains TGF-ß2 cDNA. Lane 6 - blank, Lane 7- molecular weight standards.

Figure 2A. Expression of TGFß1 and TGF2 protein in normal human lacrimal gland biopsies. (a) majority of tubuloacinar structures in all five human lacrimal gland biopsies showed immunoreactivity to a polyclonal antibody to all isotypes of TGF-ß (pan TGF-ß Ab, 40x original magnification). (b) Absence of immunoreactivity to TGF-ß2-specific antisera was noted in all lacrimal gland biopsies (400x original magnification). (c) and (d). TGF-ß1-specific antibodies produced strong immunoreactivity with epithelial cells in four or five lacrimal gland biopsies. The strongest staining with TGF-ß1 antibodies was noted in the apical secretory portion and lumens of acinar epithelial complexes ((c) - 100x, (d) -100x original magnification). (e) and (f). In sections where entire Tubuloacinar structures were visualized (asterisk), TGF-ß1 staining appeared stronger in acinar than ductal epithelia ((e) imunofluorescent staining, (f) - phase, 100x original magnification).

Figure 2B. Expression of TGFß1 and TGFß2 protein in cultured human lacrimal gland acinar epithelia. The cytoplasm of cultured human lacrimal gland epithelia stained with both TGF-ß1 (top figure) and TGF-ß2 (bottom figure) antisera (100x original magnification).

Figure 3. Results of ELISA for TGF-ß1 and TGF-ß2 in supernatants (spnt) from human lacrimal gland acinar epithelial cultures and control media. TGF-ß1 [] in culture supernatants were significantly greater than media or TGF-ß2 (*0.169ng/ml±0.021)in culture supernatants ($P<0.05$)

Figure 4. Growth inhibitory effects of native human tears in mink lung epithelial cell bioassay.

Figure 5. Concentration of TGFß in native tears treated with various physicochemical techniques.

Figure 6. Growth inhibitory effects of human tears following acidification or treatment with n-acetylcysteine ("MUCOSIL™", DEY Laboratories, Napa, CA) and heating.

Figure 7. Effect of TGFß isotype specific neutralizing antisera on antiproliferative effects of human tears.

Figure 8. Results of ELISA for TGF-ß1 and TGF-ß2 for human tears. TGF-ß concentration in tears is 0.521 ng/ml +0.321. Tear TGF-ß1 concentrations were significantly greater than TGF-ß2 ($P<0.05$).

Figure 9. Western blot of native tears treated with n-acetylcysteine and heating, showing pro-TGF-ß binding to high MW complexes (about 1000 kD, probably mucins), and monomeric TGF-ß. Lane 1 purified TGF-ß1. (R&D), monomer band is present at approximately 12.5kD (arrowhead); Lane 2. blank; Lane 3. native tears - a high molecular weight band (approximately 100kD asterisk) is noted; lanes 4-6: tears treated with n-acetylcysteine and heating (lane 4), acidification with HCl (lane 5), and acidification followed by reduction with DTT (lane 6). Two bands of immunoreactivity were noted with these specimens, a stronger band at approximately 110kD, the size of the pro-TGF-ß complex (LAP plus

cytokine, star) and a weaker band of the same size as monomeric TGF-ß (approximately 12.5 kD. arrowhead)

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, tear replacement compositions containing TGFß, where TGFß is either TGF-ß1 or TGF-ß2 or a combination thereof, by way of non limiting illustration, be applied to the eye in animals and humans as a drop or within ointments, gels, liposomes, or biocompatible polymer discs or pellet. They can be attached to, carried by and/or contained within contact lenses that are placed on the eye. In general, it is desired that the mode of application be such that the composition enters the tear film or otherwise makes contact with the surface of the eye.

Further in accordance with the invention, a replacement tear composition is made by combining TGFß with a physiologically acceptable carrier. Preferrably, the preparation will be unit dose, refrigerated, with or without preservative. The composition may also contain a physiologically compatible ophthalmic vehicle as those skilled in the art can select using conventional criteria. The vehicles may be selected from the known ophthalmic vehicles which include but are not limited to water, polyethers such as polyethylene glycol 400, polyvinyls such as polyvinyl alcohol, povidone, cellulose derivatives such as carboxy methylcellulose, methylcellulose and hydroxypropyl methylcellulose, petroleum derivatives such as mineral oil and white petrolatum, animal fats such

as lanolin, vegetable fats such as peanut oil, polymers of acrylic acid such as carboxylpolymethylene gel, polysaccharides such as dextrans and glycosaminoglycans such as sodium hyaluronate and salts such as sodium chloride and potassium chloride, calcium chloride, magnesium chloride, zinc chloride, and buffer such as sodium bicarbonate or sodium lactate. High molecular weight molecules can also be used, such as mucins.

Preferred preservatives are physiologically compatible and do not inactivate TGFß or other peptides or cytokines present in the composition. Preservatives include but are not limited to alcohols such as chlorobutanol, and benzalknonium Cl and EDTA, though other appropriate preservatives known to those skilled in the art may be used.

In a preferred embodiment, the concentration of TGFß in the tear solution is from 250 pg/ml to 12.5 ng/ml, preferably 200 pg/ml to 12.0 ng/ml. Active TGFß concentrations in human tears range from 250 pg/ml to 12.5 ng/ml (mean 3.83 ng/ml). There appears to be a total latent TGFß concentration of approximately 30 ng/ml in tears. Ideally, therapeutic TGFß should be administered bound to its natural carrier or binding protein(s) in tears. At the present time, these appear to be mucins because immunoreactivity of TGFß in native tears is at a high molecular weight (approximately 1000 kD), the molecular weight of tear mucins. Data suggests that most TGFß in tears is in the proform (approximately 110 kD). Typically, this proform is converted to the active from by proteolytic enzymes such as plasmin. Plasminogen activator is normally found in human tears. It is likely that concentrations of this

protein are reduced in patients with aqueous tear deficiency. Therefore, it may be necessary to use purified (lyophilized) active TGFß. The source of this cytokine is not essential. It could be purified from platelets (a rich source of TGFß1) or recombinant TGFß could be used. Alternatively, cultured lacrimal gland acini could serve as the source of TGFß. Other lacrimal gland produced tear constituents which may be desirable to add to physiologic tear replacements include, lactoferrin, 1-3 g/L (Kijlstra, A. et al. (1983), Br. J. Ophthalmol. 67:199-202), lysozyme, 0.5-4.5 g/L, and Tear specific pre-albumin, 0.5-1.5 g/L (Berman, E.R. Biochemistry of the Eye, Ed. C. Blakemore, Plenum Press, New York, 1991), mucins, and epidermal growth factor (EGF) 0.75-9.7 ng/ml (van Setten, G.B. et al. (1989) Graeffe's Arch. Clin. Exp. Ophthalmol. 22:184-187; Ohashi, Y. et al. (1989) Invest. Ophthalmol. Vis. Sci. 30:1879-1882), and Vitamin A, 16 ng/ml of retinol (Vitamin A is present in tears as retinol but would need to be added to tear replacement as trans retinoic acid) (Ubels, J. L. and Mac Rae, S. M. (1984) Current Eye Res. 3:815-822).

The following examples are presented to illustrate further various aspects of the present invention, but are not intended to limit the scope of the invention in any respect.

EXAMPLE 1

Production of TGFß by human lacrimal gland epithelia.

We have recently evaluated normal human lacrimal gland biopsies and cultured human lacrimal gland acinar epithelia (Yoshino, K. et al. Sjorgren's Syndrome- Proceedings of the Fourth International Symposium, 1993. Ed. M. Homma, S. Sugai, T. Tojo, N. Miyasajka and M. Akizuki, Kugler Publications, 1994, Amsterdam/New York) for expression of TGFß1 and TGFß2 mRNA and protein using RT-PCR, sELISA and immunohistochemisty, techniques known in the art (Ji, Z. et al. Invest. Ophthalmol. Vis. Sci. (1994 ARVO abstracts) 1994; 34: 1792). TGFß1 and ß2 mRNA expression was found in both lacrimal gland biopsies and acinar cultures (Figures 1A and 1B). In lacrimal gland biopsies, immunoreactivity to TGFß1 but not TGF-ß2 was detected in the secretory portion of the lacrimal gland acinar epithelia adjacent to the lumen by immunohistochemistry (Figure 2A). The cytoplasm of cultured acinar epithelia showed immunoreactivity to both TGFß1 and ß2 specific antisera (Figure 2B). TGFß1 was detected in supernatants of lacrimal gland acinar cultures in significantly greater concentrations (0.5-2 ng/ml) than the control (culture media on substrate) by sandwich ELISA (sELISA, Figure 3). Furthermore, stimulation of cultured human lacrimal gland acini with 0.01mM carbachol (a cholinergic agonist) resulted in at least a 30% increase in TGFß1 concentrations in the supernatants. These experiments indicate that TGFß is produced and secreted by human lacrimal gland

acinar epithelia, and that this secretion may be enhanced by cholinergic stimulation.

EXAMPLE 2

TGFß in human tears.

We recently evaluated human tears for TGFß using the CCL-64 mink lung epithelial cell (MLEC) growth inhibition assay, a conventional assay for the detection of TGFß, and sELISA (Danielpour D. et al. (1989) Cell Physiol. 138:79-86). Native human tears produced an anti-proliferative effect in the MLEC assay; however, a flat growth inhibition curve with rapid loss of anti proliferative activity after 3 to 7 serial dilutions was noted with native tears (Figure 4). Heating and acidification, two physicochemical techniques previously reported to activate latent TGFß increased the concentration of TGFß in human tears calculated at the midpoint of the growth inhibition curves (Figure 5). Furthermore, incubation of human tears with n-acetylcysteine ("MUCOCIL"™, DEY Laboratories, Napa, CA), a mucolytic and reducing agent, followed by heating at 80°C for 8 minutes appeared to release latent TGFß in tear samples, compared to tears treated by heating alone (Figure 6). Following this treatment, a growth inhibition curve with a slow decay of the growth inhibition activity as tear specimens were serially diluted was obtained that resembled the curve obtained with serially diluted purified human platelet TGFß1 (Figure 6). The anti-proliferative effect of human tears in the MLEC assay could be inhibited by pre-incubation with TGFß1 neutralizing anti-sera but not by TGFß2-specific antisera (Figure 7).

The presence of TGFß in human tears was confirmed by TGFß1 sELISA. TGFß1 was not detected in native tear samples by sELISA; however, pre-treatment of human tears with n-acetylcysteine followed by heating resulted in an average detectable tear TGFß1 concentrations of 45 ng/ml (range 19.99-67.7 ng/ml). TGFß2 was detected in human tears by sELISA at very low concentrations (521 pg/ml with a range of 316-891 pg/ml) compared to TGFß1 ($p<0.05$).

SDS-PAGE and immunoblotting experiments were performed to confirm the molecular weights (MW) of TFGß complexes in human tears.

EXAMPLE 3

Western blot analysis

Western blots were preformed as follows. Kaleidoscope pre-stained molecular weight standard was purchased from Bio-Rad (Richmond, CA). Human platelet TGFß1, rabbit anti-pan isotype TGFß was purchased from R&D Systems, Inc. Anti-rabbit and anti-goat IgG-POD were purchased from Boehringer Mannheim (Indianapolis, IN).

Fresh human tear specimens were activated by the following methods: (1) heating at 80°C for 7 minutes and immediately placed on ice; (2) diluted 1:1 with 10% N-acetylcysteine "MUCOCIL"™, DEY Laboratories, Napa, CA) then heated at 80°C for 7 minutes and immediately placed on ice, (3) acidification by adjusting pH to 2 with 1N HCl and incubating at room temperature for 1 hour. The pH was then neutralized with one NaOH, (4) acidification, then reduction by addition of 5ul of 1 M dithiothreitol (DTT). All activated specimens

were then added to 2X sample buffer and boiled at 100°C for 3 minutes.

Mini-protein II 4-20% Ready gels were used for SDSpolyacrylamide gel electrophoresis (SDS-page) and were purchased from Bio-Rad. Running buffer contained Tris/glycine with SDS. Electrophoresis was performed at constant voltage (125V) in a Bio-Rad mini-protein II electrophoresis cell until the dye marker had reached the bottom of the gel. Electrophoretic transfer on to PVDF membrane (Millipore, Beford, MA) was performed with a Bio-Rad Trans-Blot cell. Transfer buffer consisted of glycine/ethanolamine and 20% methanol. Prior to transfer, the PVDF membrane was pre-wet in 100% methanol, rinsed with distilled water and immersed for 15 minutes in buffer. Transfer was performed at 20V overnight. After electroblotting, membranes were stained with Pouceu S (Sigma) for 2 minutes, then rinsed with water and air dried.

Immunodetection was performed using a Bio-Rad chemiluminescent detection kit. The PVDF membrane was wet with 100% methanol, then rinsed with distilled water. The membrane was then incubated for 1 hour in blocking solution (1% blocking reagent in TBS) on a shaking incubator. The membrane was then incubated for one hour with primary antibody diluted in 0.5% blocking solution. Dilution of Pan-TGFI3 antibody was 1:2000 (1μg/μl). The membrane was then washed twice in TBST for 10 minutes each, then washed twice with 0.5% blocking solution. The membrane was then incubated for 1 hour with POD-conjugated secondary antibody diluted 1:1000 in 0.5% blocking solution. The membrane was then washed four times with TBST for 15 minutes

each. Excess buffer was then drained from the washed membrane, and it was placed in a staining dish and incubated for 30 minutes at room temperature with a mixture of solutions A and B (diluted 1:100 and incubated for 30 minutes at room temperature prior to addition). Approximately 125 µl/cm sq. was added to the membrane container and incubated for 1 minute. The wet membrane was immediately placed into a plastic hybridization bag and the bubbles were removed. The membrane (protein side up) was placed into a film cassette against a sheet of X-ray film (X-Omat, Kodak, Rochester, NY) and was exposed for 1 minute, then developed. Either no immunoreactive bands or high MW bands (> 250,000 kD) were observed in native or heat treated tears. Treatment of tears with n-acetylcysteine and heating, HCl, or HCl plus DTT resulted in immunoreactive bands at 110kD, and 12.5 kD using TGFß specific antisera (Figure 9). These bands correspond to the published MWs of pro-TGFß complexes and monomeric TGFß.

Taken together, these results indicate than native human tears contain a small amount of biologically active TGFß (approximately 3.8 ng/ml), and a greater amount of latent TGFß that can be released by a variety of physiochemical techniques. TGFß1 is the predominant isoform in tears. Our finding of TGFß1 production by human lacrimal gland secretory acini coupled with the previously reported relative lack of immunoreactivity of human ocular surface epithelia for TGFß (Pasquale, L.R. et al. Invest. Ophthalmol. Vis. Sci. 1993;94:23-30) (only superficial limbal epithelia were positive) suggests

that some, if not the majority, of TGFß in human tears may be produced by the lacrimal gland.

WHAT IS CLAIMED IS:

1. A topical ophthalmic composition suitable for administering to the eye comprising a pharmaceutically effective amount of a cytokine and a pharmaceutically acceptable carrier.

2. The composition according to claim 1 wherein the cytokine is transforming growth factor beta (TGFß).

3. The composition according to claim 2 wherein active TGFß is present in the amount of 250 pg/ml to 12.5 ng/ml.

4. The composition according to claim 2 which further comprises other growth factors selected from the group consisting of EGF, ßFGF, and retinoic acid.

5. The composition of claim 1 wherein the composition is in the form of a gel or a liquid.

6. A method of ameliorating a tear dificiency condition comprising the steps of administering to the ocular surface a pharmaceutically effective amount of an ophthalmological composition comprising a pharmaceutically effective amount of TGFß in a pharmaceutically acceptable carrier.

7. The method according to claim 6 for the tear deficiency condition is dry eye.

8. The method according to claim 7 wherein said dry eye disease is Sjogren's Syndrome.

9. The method according to claim 7 wherein the dry eye condition results from a condition selected from the group consisting of hyperproliferation, squamous metaplasia, loss of goblet cells, and abnormal terminal differentiation.

10. A cell culture comprising human lacrimal gland cells which mimic *in vivo* lacrimal gland cells and which can be modulated to affect their capability to synthesize and secrete tear proteins.

11. A process for measuring effect of an agent or a composition as to its ability to stimulate or inhibit tear secretion, said process comprising:

adding a compound or agent of interest to a culture containing the cells of claim 10, and

measuring a predetermined parameter of the cells and comparing this to a baseline value to determine the degree of activity or effect.

12. In a pharmacological preparation for the replacement of tears wherein the improvement comprises TGFß in a pharmacologically acceptable carrier.

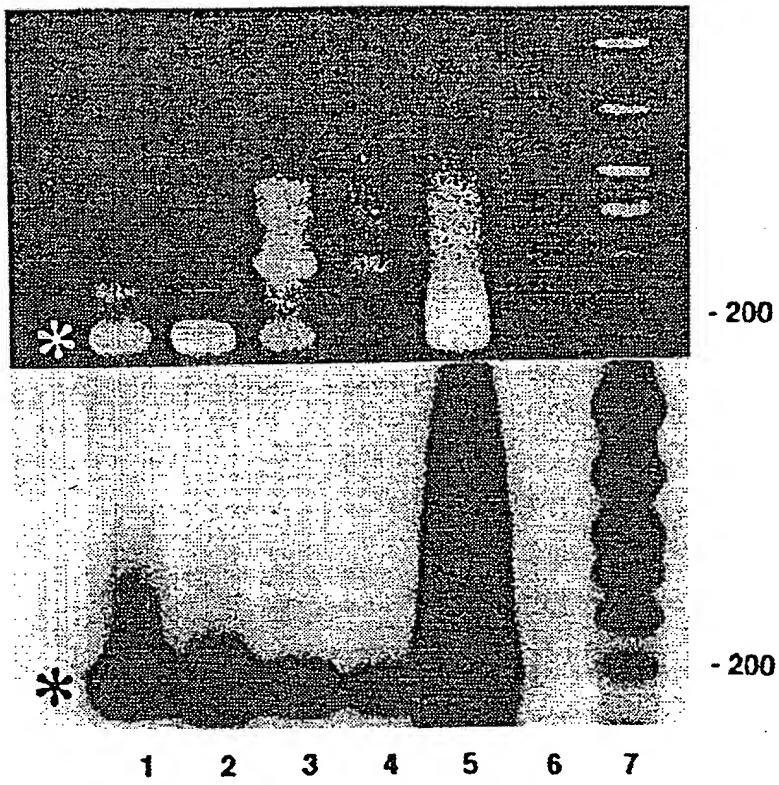


FIG. IA

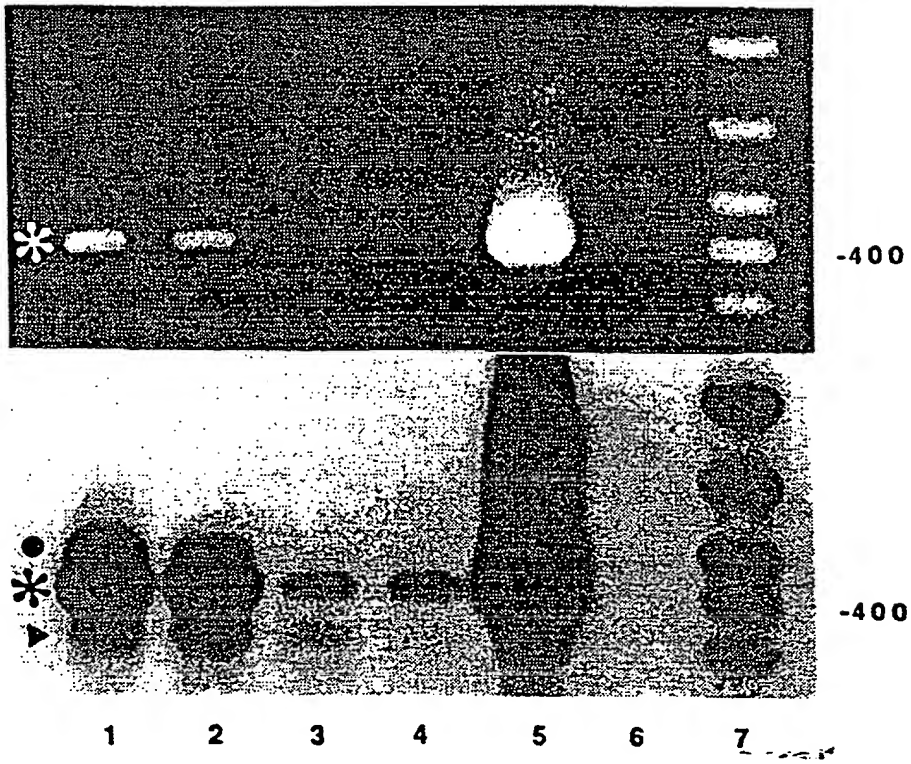


FIG. IB

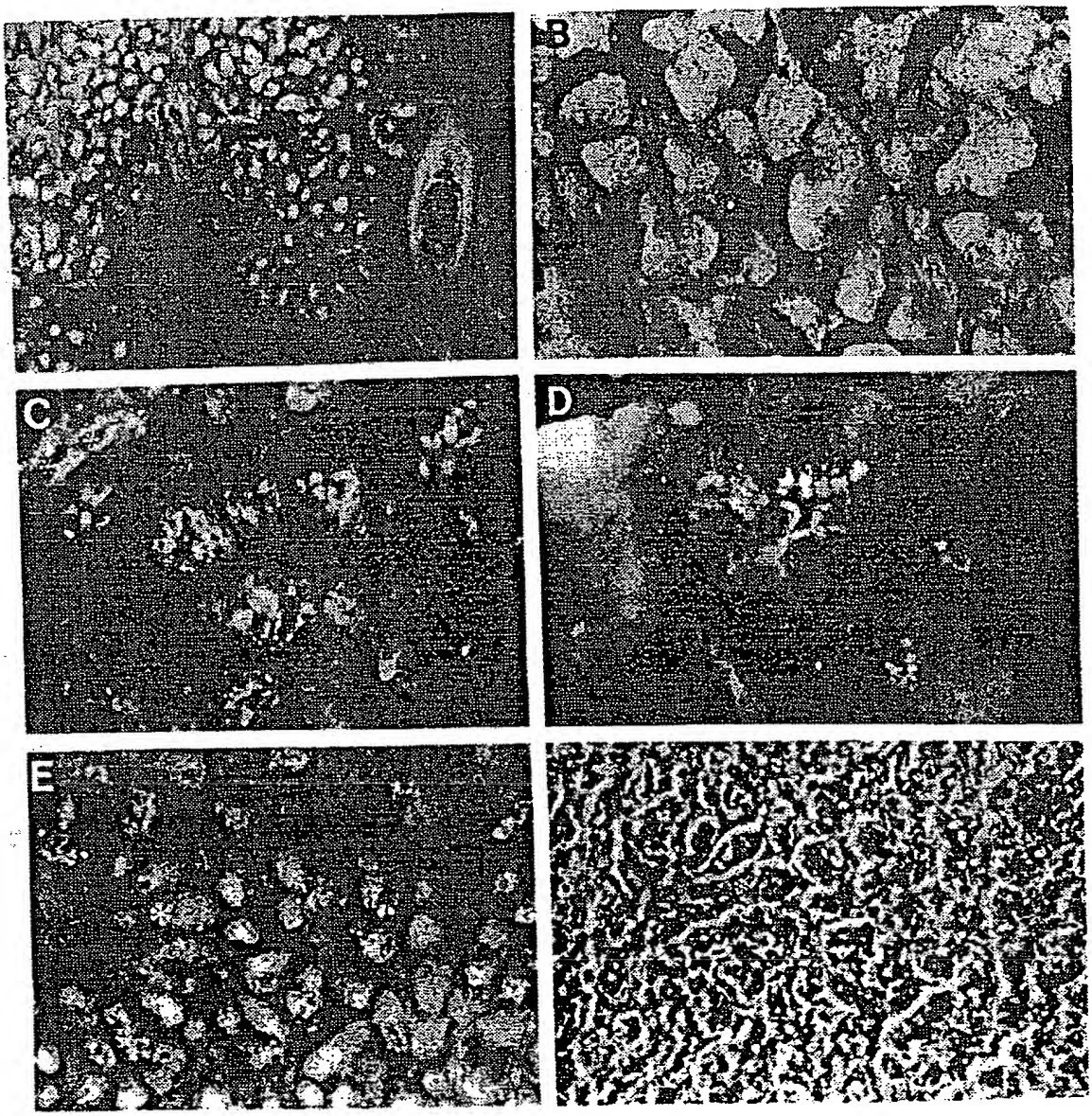


FIG. 2A

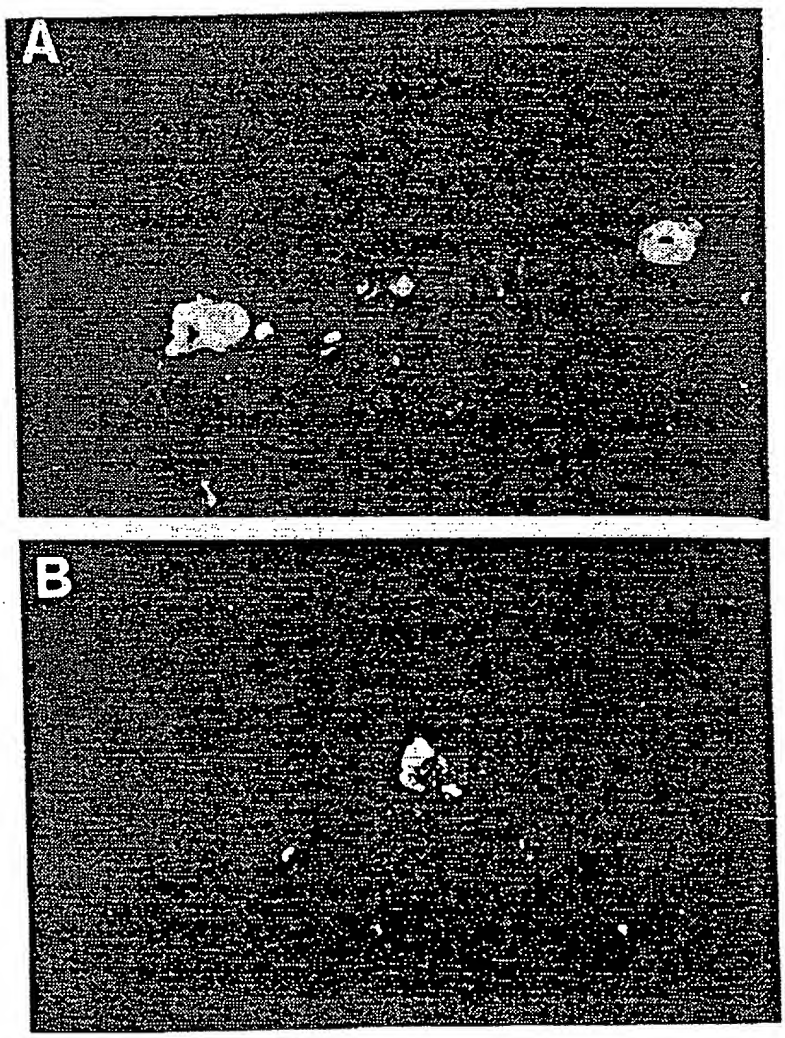


FIG. 2B

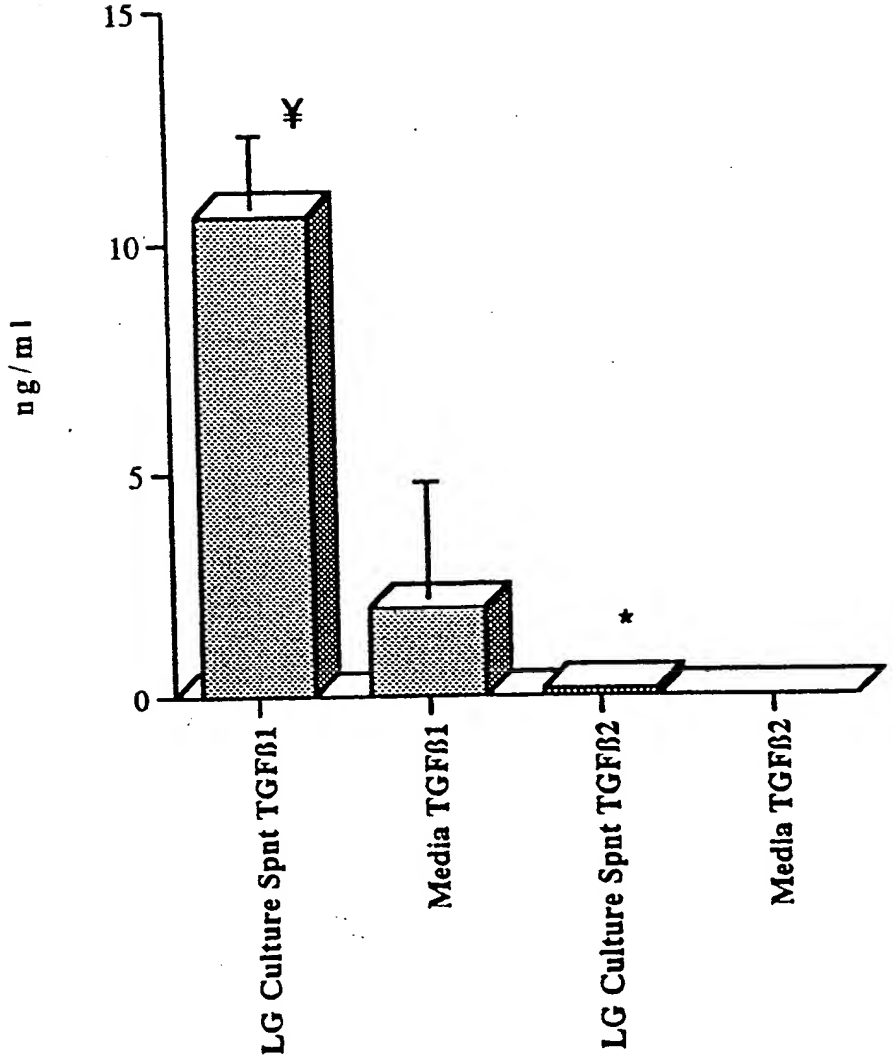

15
10
5
0
ng/ml
¥
*
LG Culture Spnt TGFß1
Media TGFß1
LG Culture Spnt TGFß2
Media TGFß2

FIG. 3

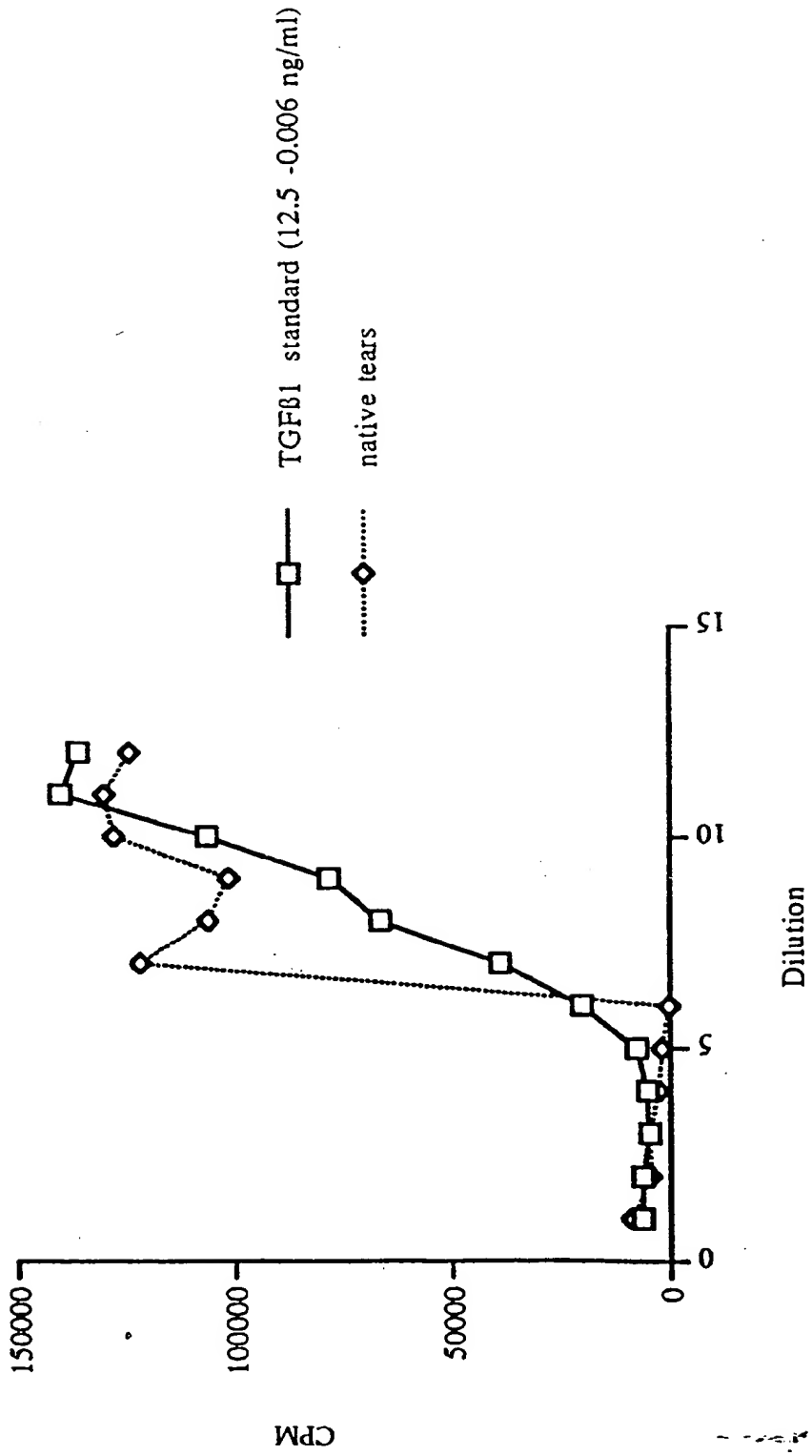


FIG. 4

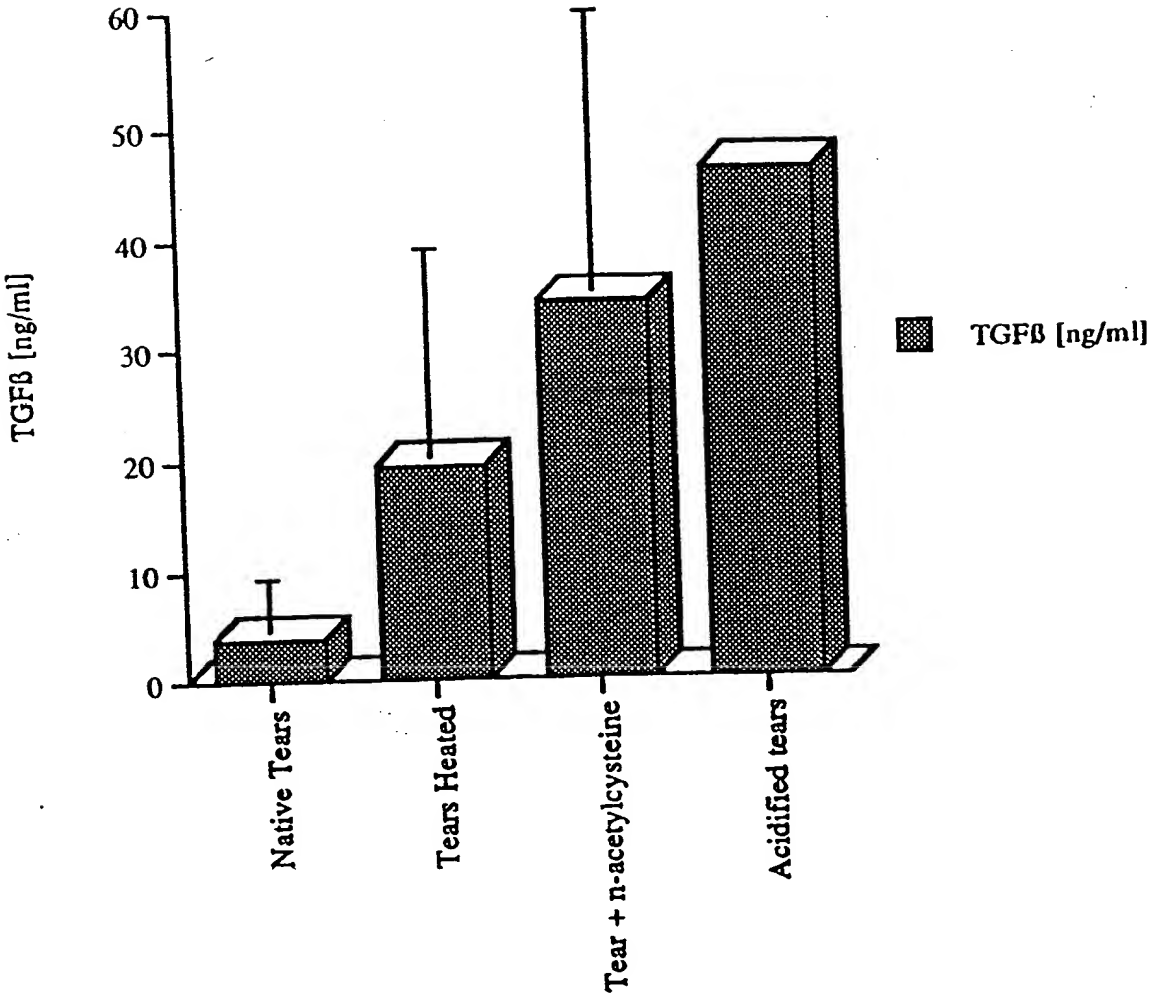

60
50
40
30
20
10
0
TGFß [ng/ml]
Native Tears
Tears Heated
Tear + n-acetylcysteine
Acidified tears
TGFß [ng/ml]

FIG. 5

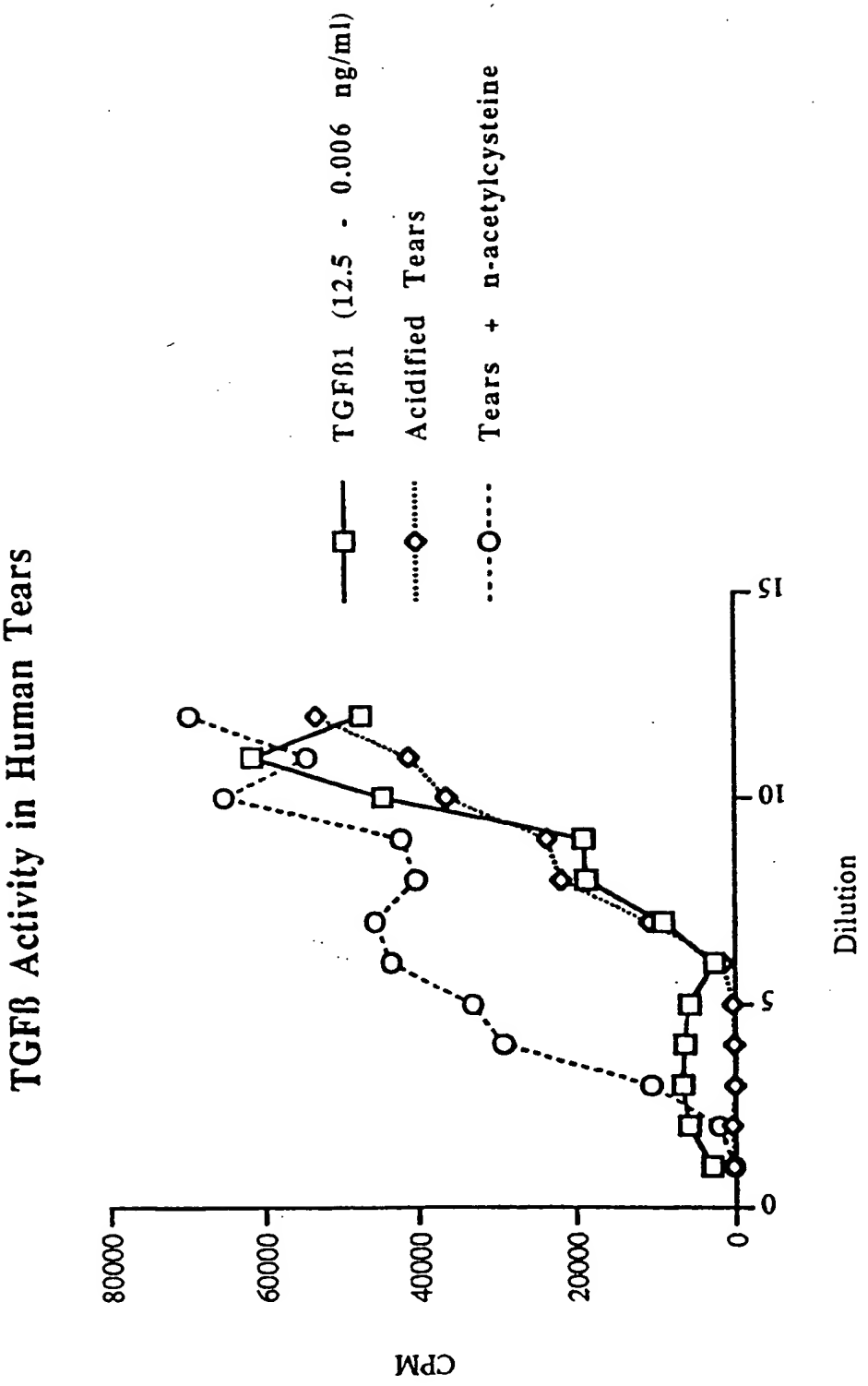


FIG. 6

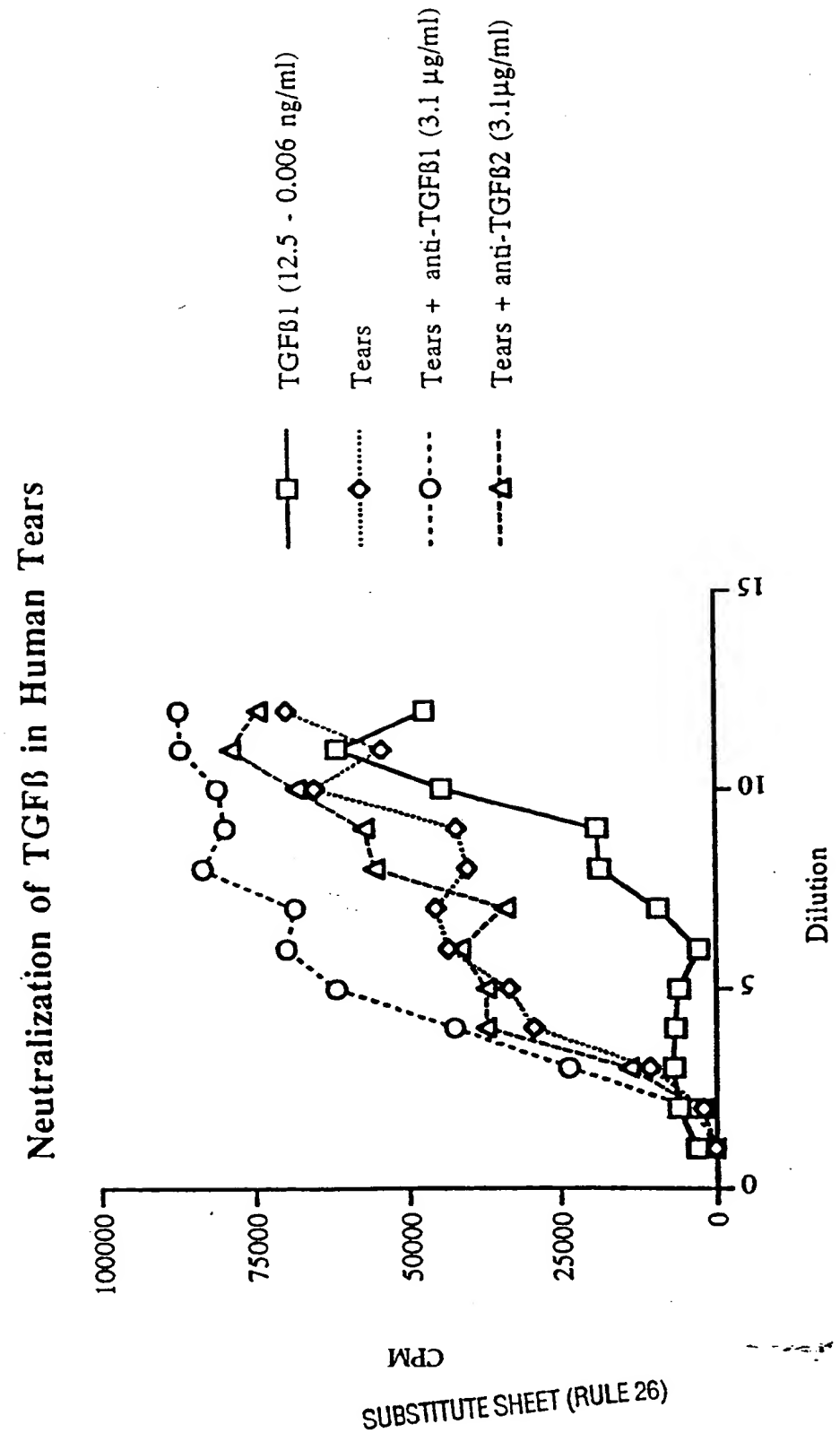

Neutralization of TGFß in Human Tears
TGFß1 (12.5 - 0.006 ng/ml)
Tears
Tears + anti-TGFß1 (3.1 µg/ml)
Tears + anti-TGFß2 (3.1µg/ml)
CPM
100000
75000
50000
25000
0
0
5
10
15
Dilution

FIG. 7

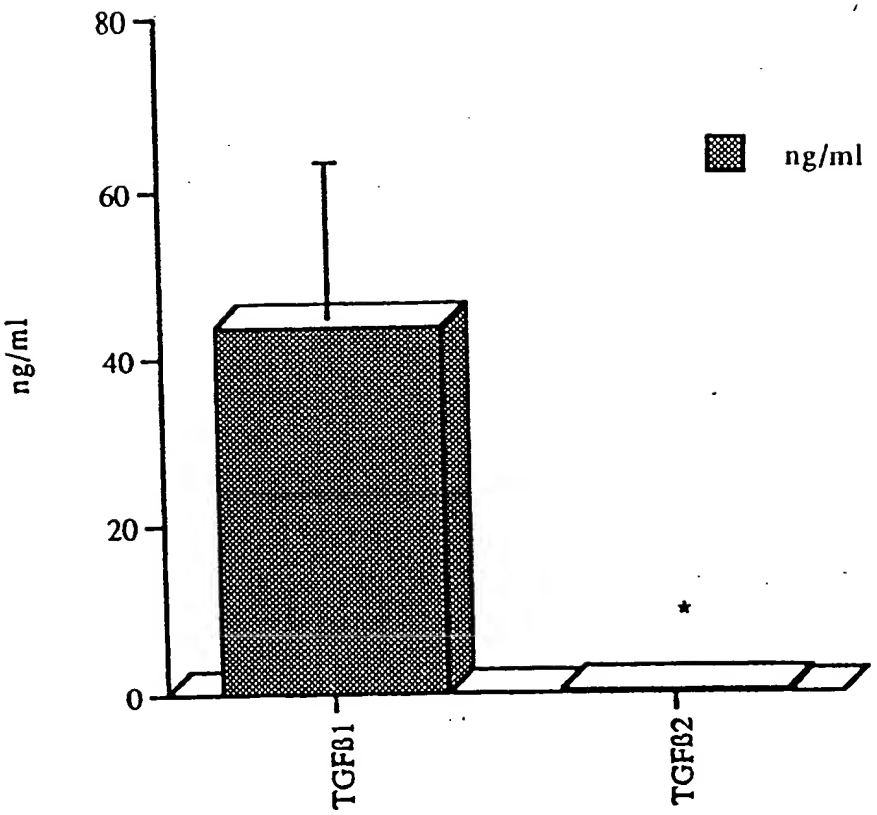
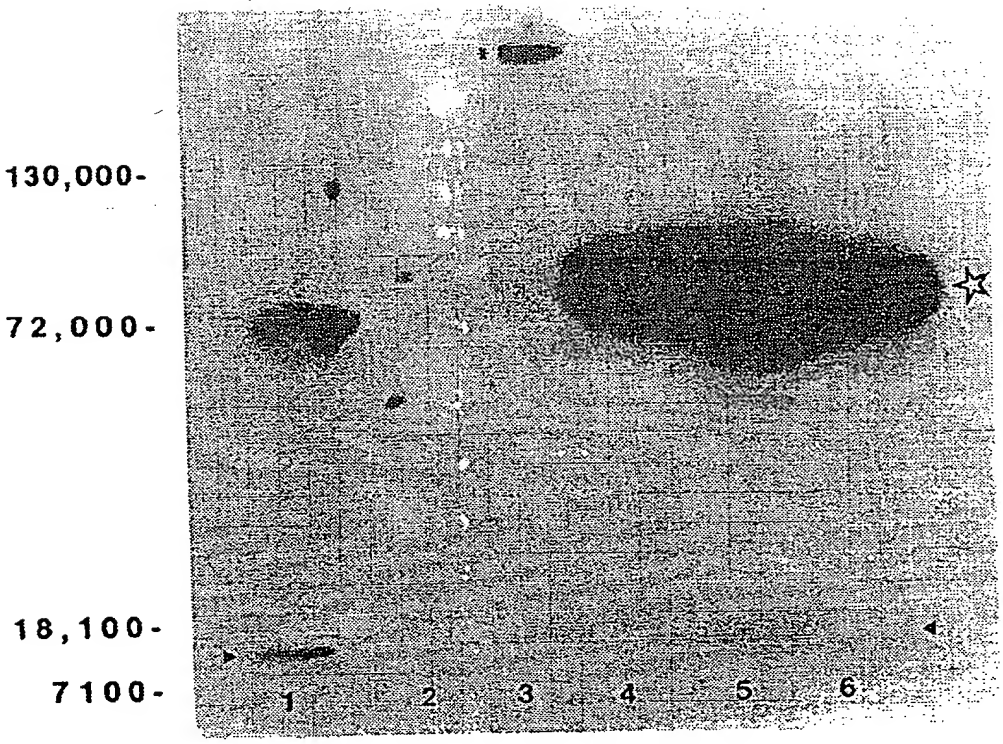


FIG. 8



Immunoblot of human tears for TGF-β

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05015

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/19
US CL :514/2, 912
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,212,162 (MISSEL et al.) 18 May 1993, see the entire patent.	1-5 and 12
Y	Proceedings of the Fourth International Symposium, issued 11-13 August 1993, (Amesterdam/New York), "The characterization of human lacrimal gland acinar and ductal epithelia in various culture system", pages 1-4, see entire document.	10, 11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 JULY 1995	Date of mailing of the international search report 22AUG 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ZOHREH FAY aco Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*